

## Immortalization of human adult prostatic adenocarcinoma cells by human papilloma virus HPV16 and -18 DNA

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### Abstract

Primary prostate epithelial and prostate adenocarcinoma cells cultured in serum-free medium grew for up to 10 passages before senescence. Cells from prostate adenocarcinoma of a 55-year-old patient without lymph node involvement were transfected with plasmids containing recombinant human papilloma virus HPV16 or HPV18 DNA and the selectable neomycin-resistance gene. After G-418 selection, cells underwent crisis, and surviving cells infected with retroviruses encoding the HPV18 E6/E7 genes (HPV-PAC1), transfected with a head-to-tail dimer of the complete HPV16 genome (HPV-PAC2), or transfected with HPV18 E6/E7 early genes (HPV-PAC3) were established. HPV-PAC1 and HPV-PAC2 cultures appeared morphologically similar to primary cultures even after 40 passages. However, HPV-PAC2 cultures had a clonal morphology. All lines were positive for cytokeratin 18, had acquired vimentin expression, and contained either HPV16 or HPV18 sequences integrated into host DNA. None was tumorigenic in nude mice or formed colonies in soft agar. These cells did not secrete prostate specific antigen nor respond to androgen although tamoxifen inhibited the growth of the cells. Immunohistochemistry showed no evidence of p53 overexpression. Further characterization of these cell lines and examination of their response to chemotherapeutic agents may provide relevant information for the study of hormone-independent PC.

**Keywords:** Human prostatic cell; Immortalization; Human papilloma virus DNA

### 1. Introduction

Prostatic carcinoma is the most frequently diagnosed malignancy and a major cause of cancer deaths for males in the United States second only to lung cancer [1]. The incidence of prostate carcinoma increases most rapidly with age [2,3]. Although potentially curable in its early stages, metastatic prostatic

cancer is incurable, with temporary remission commonly achieved by hormonal therapy. Despite the high morbidity and mortality of the disease, the molecular events involved in the development and progression of prostate cancer are still poorly defined. Mutational activation of the ras oncogenes [4] and inactivation of the p53 and retinoblastoma tumor suppressor genes [5–7] have been observed. The frequency of these genetic events, however, appears to be rather low, even in advanced stages of the disease.

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Therefore, a growing need to gain further understanding of the genetic pathways underlying prostate tumorigenesis exists.

Epidemiological and clinical studies implicate human papilloma virus (HPV) in the etiology of a variety of squamous epithelial tumors [8,9]. HPV16 and -18 account for approximately 70% of HPV positive cervical carcinoma. Unlike DNA from HPV-6 and -11, which induce benign genital tumors [10,11], DNA from HPV16 and -18 can immortalize primary keratinocytes in culture and this difference in transformation ability appears to be due to biological differences in their E6 and E7 genes [12,13]. The E6 and E7 proteins of malignancy-associated HPV but not benign-associated HPV combine efficiently with the tumor suppressor proteins of the p53 and retinoblastoma genes, respectively [14,15]. Differences also exist between HPV16 and HPV18 in that the latter is 10-50-fold more active in immortalizing keratinocytes in vitro [16], and these differences map predominantly to the viral enhancer region [17]. Reports show that HPV18-immortalized keratinocytes spontaneously progressed to malignancy [18].

One of the most characteristic features of HPV infectivity is site and cell type specificity. The current evidence does not implicate HPV in human prostate cancer. While earlier studies claimed a high prevalence of HPV16 in benign prostatic hyperplasia (BPH) and prostate adenocarcinoma [19-21], more recent evidence strongly argues against any causative involvement of HPV in human prostate tumorigenesis [4,22,23]. HPV16 and 18 DNA was used in the present study to immortalize human prostate epithelial (HPE) cells to provide a model for investigating the pathways in prostate tumor progression.

## 2. Materials and methods

### 2.1. Prostate cell culture

Human prostatic tissue was obtained after prostatectomy from patients diagnosed with primary adenocarcinoma. Tissue was minced to 1-2 mm and incubated ON at 37°C in collagenase 200 U/ml (Worthington) in HBSS, with 5% FBS. The digest was triturated by repeated pipetting and centrifuged. The pellet was washed with HBSS, resuspended and plated in 100 mm culture dishes. The culture media

was a modification of MCDB153-LB [24]. When prostate epithelial cell colonies reached 0.5-1 cm<sup>2</sup>, the primary culture was passaged with trypsin/EDTA (0.2%/0.02%). Cells were plated at a density of approximately  $0.5 \times 10^6$  in 100 mm dishes and used for HPV transfection.

### 2.2. Recombinant plasmids

The plasmid pMHPV16d, a head to tail dimer of HPV16 DNA [25] inserted into the BamHI site of the plasmid pDMTneo [26] and recombinant retroviruses encoding HPV18 E6 and E7 genes have been described [27,28].

### 2.3. DNA and RNA analysis

High molecular weight DNA was isolated using DNA Stat-60 (Tel-test B, Inc.). Ten micrograms of purified DNA was digested with restriction endonucleases BamHI or EcoRI and DNA was separated by electrophoresis in 0.8% agarose gels and transferred to Gene Screen membrane (New England Nuclear).

DNA probes used for hybridization analysis were the BamHI fragment of HPV16 or the EcoRI fragment of HPV18, purified from the respective vectors. DNA was labeled to high specific activity ( $5 \times 10^8$  dpm/ $\mu$ g of DNA) by using [<sup>32</sup>P]dCTP and the multiprimer DNA labeling kit purchased from Amersham (Amersham Corp., Arlington Heights, IL). Membranes were hybridized according to the protocol described by Church and Gilbert [29]. Prehybridization was performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA and 1% bovine serum albumin for 2-4 h at 65°C in rotating tubes. Hybridization was performed overnight in the same buffer. The filters were washed first with 2× SSC, 0.5% SDS, then with 2× SSC, 0.1% SDS at 24°C for 15 min and finally with 0.1× SSC and 0.5% SDS at 65°C for 2 h (Tm-17°C). Under these conditions HPV16 and -18 do not cross hybridize. Autoradiography was performed by exposing the filters to Kodak XAR-5 film at -70°C with an intensifying screen for 24 h. To estimate HPV copy number, an amount of plasmid equal to 0.5, 2.5 or 5 copies per cell was mixed with salmon sperm DNA and also analyzed by Southern blot. The 8 kb position indicates linear HPV.

Total RNA was extracted from the cells with RNA State-60 (Tel-test B, Inc.). RNA electrophoresis was performed in 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to a Gene-Screen filter and hybridized as described for DNA.

#### 2.4. Immunohistochemistry

Cells were fixed in culture dishes with ice-cold methanol/acetone (2:1) for 15 min, air-dried and used for immunostaining. The antigens tested were identified by indirect immunofluorescence or immunoperoxidase labeling. Mouse monoclonal antibodies were used to detect cytokeratin 18 (Sigma), Vimentin (Sigma), prostatic specific antigen PAS (Sigma), prostatic acid phosphatase, PAP (Sigma), and p53 (Oncogene Science). After 1 h incubation at 24°C with the appropriate dilution of cytokeratin antibodies, rabbit anti-mouse immunoglobulin was added for 30 min.

#### 2.5. Growth curves and androgen binding

The effect of the anti-estrogen tamoxifen on HPE growth was determined on cultured cells 24 h after attachment. The medium was replaced with medium containing 0.01, 0.1, 1, or 10  $\mu$ M tamoxifen. Tripli-

cate cultures were incubated for 7 days and medium was changed every 48 h. Cells were trypsinized and counted with a hemacytometer. Viability was determined with 0.4% Trypan blue. To determine the presence of androgen receptors, intact cells were assayed for specific binding of the synthetic androgen, [ $^3$ H]mibolerone (New England Nuclear). The cells were cultured in 12-well culture plates in SFM-keratinocyte basal medium for 72 h. Medium containing labeled mibolerone ( $10^{-12}$ – $10^{-7}$  M) was added for 1 h at 37°C. Intracellular radioactivity was solubilized with 0.5 M NaOH after washing the wells three times with ice-cold TCA. For growth assays with mibolerone ( $10^{-12}$ – $10^{-7}$  M) the total number of cells were counted after 7 days.

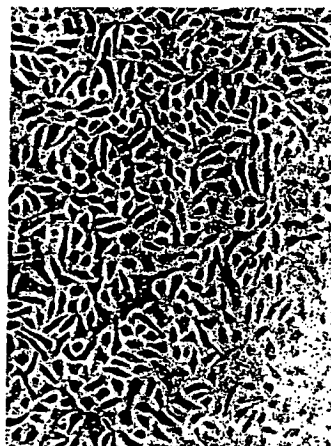
### 3. Results

Primary cultures of HPE cells derived from normal prostate tissue migrated from attached acini (Fig. 1, passage 0) and reached confluence within 1 week (Fig. 1, passage 1). With subsequent passages, the cells became larger and more flattened (Fig. 1, passage 5), appeared to senesce, and could no longer be subcultured.

Primary cultures from prostate tissue of a patient diagnosed with prostatic adenocarcinoma (Gleason



PASSAGE 0



PASSAGE 1



PASSAGE 5

Fig. 1. Morphology of primary HPE cultures. Acinar structures 1 day after collagenase digestion of primary prostate tissue (passage 0). Confluent monolayer of epithelial cells 1 week after isolation (passage 1). Senescent cells (passage 5).

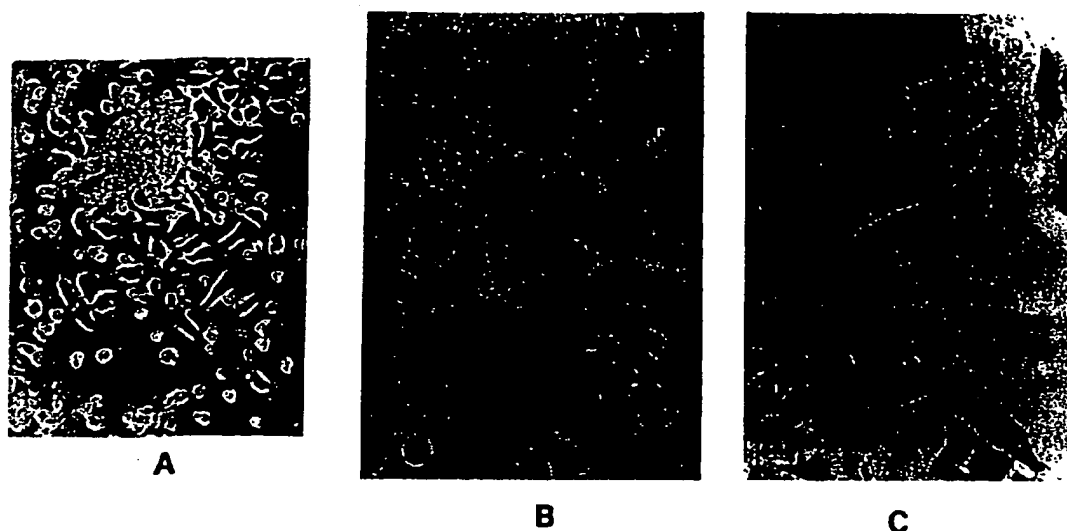


Fig. 2. Morphology of HPV transfected prostate adenocarcinoma cells. (A) Cells migrating from isolated acini. (B) HPV16d immortalized cells post-crisis exhibit rounded morphology. Cells are stained with keratin antibody. (C) HPV18 E6, E7 DNA immortalized cells exhibit a typical epithelial morphology (keratin stained). No specific staining was observed without primary antibody and in control fibroblast cultures.

grade 3 + 4 = 7) migrated from attached acini (Fig. 2A) and formed typical epithelial monolayers similar to HPE cells from normal prostate. These cells also eventually senesced (data not shown). Secondary cultures of these adenocarcinoma cells were transfected with pMHPV16d, the HPV18 E6/E7 genes or vector pdMMTneo or infected with the retrovirus HPV18 E6/E7 genes [28] and selected for resistance to the antibiotic G418 (100  $\mu$ g/ml of media) for 48 h. Cells were passaged by trypsinization once a week. Cells transfected with HPV16d continued to proliferate after passage 10 followed by a series of crises after passage 25. After 3 months, significant differences in morphology were observed (Fig 2B). The cells were smaller and almost round. Growth during crisis was very slow with doubling times of 60–90 h but after crisis (passage 68) the doubling time was 40 h. The cells have been subcultured repeatedly and no further crises have been observed. In contrast, HPV18 transfected cells did not undergo crisis with subculturing. Several HPE isolates were immortalized by HPV18 DNA, one of which is described here (Fig. 2C). These HPE/HPV immortalized cell lines grew to confluence but did not overgrow the mono-

layer and thus never lost contact inhibition. No colony formation in soft agar was observed, nor were tumors induced after subcutaneous cell injection into nude mice. The effect of tamoxifen on cell growth was examined because of the reported effects of tamoxifen in increasing TGF $\beta$  production. Tamoxifen inhibited the growth of HPE/HPV cells at concentrations greater than 1  $\mu$ M (data not shown).

HPE/HPV cells grew in SFM-keratinocyte medium and exogenous androgen did not stimulate the growth of the cells suggesting a loss of androgen response. Confirming this, specific hormone binding assays were negative. Because PSA expression is regulated by the androgen receptor, the expression of PSA and specific epithelial cytokeratin 18, vimentin, and prostatic acid phosphatase (PAP) were examined by immunocytochemistry (Table 1). Cytokeratin 18 is constitutively expressed in secretory prostatic epithelial cells and was detected in HPE/HPV 16 and 18 immortalized cells. Vimentin which is often present in cultured epithelial cells, was also present. PAP and PSA, markers of secretory prostatic cells in normal and well differentiated prostatic carcinoma, were present in the initial passages, but progressively di-

minished and were not detectable after passage 10. At passage 35 PSA was negative when analyzed by ELISA techniques (Tandem PSA kit, Hybritech Corp) which can detect 1 ng/ml of PSA. p53 protein expression was negative as expected because the E6 protein of oncogenic HPV types binds to p53 leading to increased protein turnover.

To determine whether HPV16 or 18 DNA was present in the HPV16 or 18 immortalized HPE cell lines, high-molecular-weight DNA was obtained at early (E) or late passages (L) (12-14 to 35-50). Digestion with *Eco*RI and detection with an HPV18-specific probe revealed a band from HPE/HPV18 transfected cells and control HeLa cells (Fig. 3A), indicating integration of the HPV genome. HPE/HPV16d transfected cells and control SiHa cells (containing HPV16) did not exhibit a band. However, an HPV16-specific probe detected HPV16 sequences in HPE/HPV16d transfected early and late passage cells and control SiHa cells. The HPE/HPV16d cell line was tested for resistance to G418, and results revealed loss of resistance to G418 while the HPE/HPV18 cell line retained G418 resistance.

To estimate the number of HPV 16 copies in the transfected lines, a reconstruction experiment was performed by mixing amounts of the plasmid DNA equivalent to 0.5, 2.5 and 5 HPV copies per cell with salmon sperm DNA. The DNA was digested with *Bam*HI. The copy number of HPV in the HPE/HPV cell lines was less than 1 suggesting that part of the HPV genome was lost. The state of the HPV16 sequence in HPE/HPV16d cell lines at early and late passages was similar and suggests that rearrangements did not occur during culture.

Table 1

Characteristics of HPV immortalized prostate cells

	HPE	HPE/HPV18	HPE/HPV16d
Cytokeratin 18	+	+	+
Vimentin	n.d.	+	+
PSA	±	-	-
PAP	n.d.	-	-
Androgen receptor	n.d.	-	-
Soft agar growth	-	-	-
Tumors nude mice	-	-	-
p53	n.d.	-	-

n.d., not done.

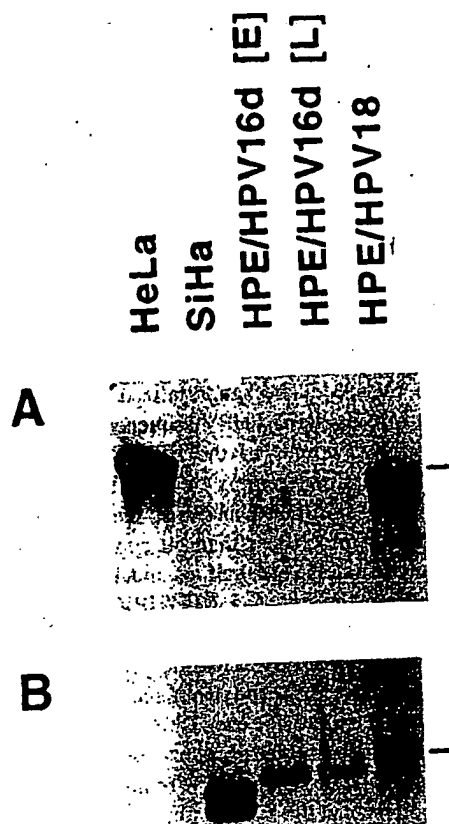


Fig. 3. Southern blot to detect HPV18 and HPV16 sequences. Genomic DNA was digested with *Eco*RI and resolved on agarose gels. (A) An HPV18-specific probe was used. HeLa cell DNA was the positive control. (B) An HPV16-specific probe was used. SiHa cell DNA was the positive control. An 8 kb *Bam*HI plasmid fragment was used as a marker and is indicated by the bar.

#### 4. Discussion

Primary adenocarcinoma prostate cells were immortalized using HPV16 and 18 DNA. The transfected HPE/HPV cell lines have now been cultured for more than 12 months and 70 passages. HPV16 or 18 DNA was responsible for the extended life-span of HPE cells since vector sequences alone were ineffective. Maintenance of HPV sequences did not depend on G418 expression as demonstrated by two of the three cell lines that lost G418 resistance.

None of the primary prostate epithelial cell isolates were immortalized with the recombinant papillomavirus DNA. In contrast, the efficiency of immor-

talization of keratinocytes is higher. This may be due to the fact that in cervix the relatively abundant basal cells are susceptible to infection by virus, and as a result of differentiation clones of infected cells can be detected by in situ hybridization. The low efficiency in prostate tissue may result from (1) fewer basal cells in the prostate, compared to the cervix, (2) the medium used in these experiments which was optimized for growth of keratinocytes and not prostate epithelial cells, or (3) the age of the donor.

HPV sequences are found in the overwhelming majority of cervical carcinoma or carcinoma-derived cell lines [30-33]. The immortalized HPE cells in this study also contained HPV sequences. HeLa cells contain 50-100 copies of the HPV18 genome and SiHa cells contain one copy of the HPV16 genome [34]. From the Southern analysis, the HPE/HPV18 cells contained much less than 50 copies of HPV18 since approximately one-half as much HeLa DNA was applied to the gel than HPE/HPV18 DNA. The HPE/HPV16 cells contained less than one copy of the HPV16 genome per cell as verified from the Southern analysis (Fig. 3B) and from reconstruction experiments with salmon sperm DNA. Our results are consistent with the hypothesis that extension of lifespan (immortalization) by HPV requires integration of the viral DNA in the host genome.

The available human prostate carcinoma cell lines PC-3 [35], DU-145 [36], TSU-pr1 [37], and LNCaP [38], are derived from metastases, and only LNCaP is androgen dependent. This cell line has a mutation at codon 877 in the androgen receptor gene, a mutation frequently seen in treatment-resistant prostate carcinoma. To study the events leading to prostate tumorigenesis, several groups have cultured immortalized prostate epithelial cells. Viral immortalization using SV40 T antigen, adenovirus E1A, or HPV18 DNA have been reported [21,39-41]. Adenovirus E1A was unsuccessful (W. Isaacs, pers. commun.). We also found HPV18 to be effective at immortalization. In addition, although HPV16 may be less oncogenic than HPV18, the current results show for the first time that the HPV16 dimer can also immortalize human prostate epithelial cells.

In humans, the standard therapy for metastatic prostatic cancer has been androgen ablation. Androgen independent prostate cancer cells, however, exist even before therapy is initiated. In animal models

androgen ablation induced by castration causes the regression of tumor due to activation of apoptosis of androgen dependent cells [42]. The effect is probably due to an enhanced expression of the transforming factor beta (TGF $\beta$ ) gene which is also a potent inhibitor of cell proliferation of many malignant epithelial cells. The HPV immortalized cell lines were sensitive to the growth inhibitory properties of tamoxifen which may be mediated by TGF $\beta$ . Tamoxifen reduced the growth of these cell lines by 90% while TGF $\beta$  reduced cell growth by 30%. New therapeutic approaches for androgen independent prostate cancer leading to a programmed cell death pathway are required. The use of agents that activate TGF $\beta$  in such therapy needs further investigation.

Effective chemotherapy specifically targeted against the androgen-independent cancer cell can be combined with other methods. Unfortunately there are presently no highly effective chemotherapeutic agents that can eliminate androgen-independent prostatic cells. HPE/HPV immortalized cell lines combined with other prostatic metastatic cell lines may be useful in examining the effects of various therapeutic agents that may be effective inhibitors of androgen-independent prostate tumor cells.

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